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Preliminary Analysis of Lipids and Fatty Acids of Green Bacteria and *Chloroflexus aurantiacus*

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The complex lipids and fatty acids of the seven type species of green bacteria and three strains of *Chloroflexus aurantiacus* were analyzed. The green bacteria contained lipids that behaved as cardiolipin and phosphatidylglycerol on thin-layer chromatography. They did not contain phosphatidylethanolamine or phosphatidylserine. Similarly, *Chloroflexus* contained lipids that behaved as phosphatidylglycerol and phosphatidylinositol on thin-layer chromatography and did not contain phosphatidylethanolamine or phosphatidylserine. The green bacteria contained glycolipids I and II of Constantopoulos and Bloch (monogalactosyldiglyceride and a galactose- and rhamnose-containing diglyceride). *Chloroflexus* exhibited galactose-containing glycolipids that behaved identically with the mono- and digalactosyldiglycerides of spinach on thin-layer chromatography, and each contained galactose as well as at least one other sugar. The fatty acids of both groups of bacteria consisted entirely of saturated and monounsaturated fatty acids. In the green bacteria, myristic, palmitic, and hexadecenoic acids predominated. In *Chloroflexus*, palmitic, stearic, and oleic acids predominated. The positions of the double bonds in the monounsaturated fatty acids of *Chloroflexus* indicated synthesis by the anaerobic pathway. The lipid analyses suggest a close relationship between the green bacteria and *Chloroflexus* and further suggest that these groups of photosynthetic bacteria are more closely related to the blue-green algae than are the purple bacteria.

The lipids of the nonsulfur purple bacteria have been rather extensively described (11). Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were found in all of the strains examined, whereas phosphatidylcholine (PC), cardiolipin (CL), and ornithine-containing phospholipids were found in some strains. The galactolipids characteristic of the chloroplasts of eukaryotic photosynthetic organisms are never found in the nonsulfur purple bacteria with the exception of a trace amount of a compound with the behavior of monogalactosyldiglyceride (MGDG) found in *Rhodospirillum molischianum* (8).

In contrast, the lipids of only one strain of sulfur purple bacteria have been described. *Chromatium*, strain D, contains PE, PG, CL, and glycolipids which may be mono-, di-, and triglycosyldiglycerides (27). The phospholipids of the green bacteria have not been described. Three strains of green bacteria were found to contain monogalactosyldiglyceride and a rhamnose- and galactose-containing glycolipid (10).

The fatty acids of the nonsulfur purple bacteria have been found to consist of saturated and monounsaturated fatty acids as well as, in one species, hydroxy-fatty acids, cyclopropane group-containing fatty acids, and branched-chain fatty acids (8, 21, 24, 30). The fatty acids of pure cultures of green bacteria have not been examined.

The other major group of photosynthetic prokaryotic organisms is the blue-green algae. Nichols et al. (20) reported the presence of PG, MGDG, digalactosyldiglyceride (DGDG), and sulfoquinovosyldiglyceride (SQDG) in two strains of blue-green algae. They found that PC, PE, and phosphatidylinositol were absent. We have previously reported the presence in 11 strains of unicellular blue-green algae and 13 strains of filamentous blue-green algae of glycolipids which when analyzed by thin-layer chromatography exhibit the R_f and staining behavior of MGDG and DGDG. Other glycolipids are also present in these strains, as are possibly SQDG and PC. PE was not found in any of the strains (14, 15; C. N. Kenyon, unpublished observations).

Recently B. K. Pierson and R. W. Castenholz (submitted for publication) described a phototrophic gliding filamentous bacterium of hot springs which they have named *Chloroflexus*

aurantiacus. This organism is related taxonomically to the green bacteria and, to a somewhat lesser extent, to the nonsulfur purple bacteria and to the blue-green algae. Because fatty acid and lipid compositions have been of considerable usefulness in describing the taxonomic position of other prokaryotic organisms, particularly those capable of photosynthesis (11, 14, 15), we decided to examine the lipids and fatty acids of three strains of *C. aurantiacus* and of each of the seven type species of green bacteria. This paper reports the results of a preliminary comparative study of the complex lipids and fatty acids of these organisms.

MATERIALS AND METHODS

Strains and conditions of cultivation. Type strains of all the species of green sulfur bacteria were grown in the laboratory of N. Pfennig (University of Göttingen) in the medium described by Pfennig (24) at pH 6.8 with 0.090 g of $Na_2S \cdot 9H_2O$, 0.04 g of ammonium acetate, 0.033 g of NH_4Cl, and 2 μg of vitamin B12 per 100 ml of medium. The strains were *Chlorobium limicola*, strain 6330; *C. limicola* forma *thiosulfatophilum*, strain 6230; *vibrioforme*, strain 6030; *C. vibrioforme* forma *thiosulfatophilum*, strain 1930; *C. phaeobacteriodes*, strain 2430; *C. phaeovibroides*, strain 2631; and *Pelodictyon luteolum*, strain 2530. The cell material from 500 ml of culture was concentrated by centrifugation, resuspended in a small amount of culture medium, and stored at 4 C until analyzed.

Three strains of *Chloroflexus aurantiacus* (J-10-fl, OK-70-fl, and OH-64-fl) were grown in the laboratory of R. Castenholz (University of Oregon). The cultures were grown in screw-cap Roux bottles in Van Baalen's medium Cg-10 (28) or medium D (6) supplemented with 0.2% yeast extract, at 45 C, with 170 f-c of illumination from a tungsten lamp, semi-aerobically, to a density of about 100 Klett units. Growth of all three strains was photoheterotrophic. Cell material of *Chloroflexus* was collected by centrifugation, washed thoroughly, and either analyzed directly or lyophilized and stored at −10 C until analyzed. The analyses of *Chloroflexus aurantiacus* were performed on two separate cultures of each strain with identical results.

Lipid extraction. Whole cells of green bacteria or *Chloroflexus* were sonicated for 1.5 or 4 min, respectively, in a 60-W MSE (MSE, Ltd., London) sonic disintegrator. The total lipids were extracted by the method of Bligh and Dyer as described by Ames (1).

Phosphate content of lipids. The phosphate content of the lipids was determined by the method of Bartlett (2).

Thin-layer chromatography of lipids. Lipids were analyzed by thin-layer chromatography on commercially prepared silica gel plates (Brinkmann Instruments, Inc., or Analtech, Inc.). Plates were activated at 100 C for 30 min before use. The solvent systems used for unidirectional development were (i) chloroform-methanol-acetic acid-water (85:15:10:3.5, vol/vol) (20); (ii) chloroform-methanol-7 N ammonia (60:35:5, vol/vol) (19); and (iii) chloroform-methanol-acetic acid (65:25:8, vol/vol) (19).

The solvent systems used for two-dimensional thin-layer chromatography were, in the first direction, chloroform-methanol-water (65:25:4, vol/vol), and in the second direction, diisobutylketone-acetic acid-water (80:55:15, vol/vol) (modified from Lepage [17]). The plates were dried for 30 min in air between developments. After both developments, the plates were dried thoroughly under vacuum.

All lipids were visualized by exposure of each plate to iodine vapor. After removal of the iodine, the following stains were used. PE and phosphatidylserine (PS) were visualized with ninhydrin (26). Glycolipids were visualized with diphenylamine (13). Phospholipids were visualized with the molybdate spray described by Vaskovsky and Kostetsky (29). PC was visualized with the Dragendorff reagent (4). The presence of PG was confirmed by staining with the acetylacetone spray of Schwartz (25).

The lipids of the green bacteria were identified by comparison of their R_f values in all four of the above solvent systems with the R_f values of known standards. Standards were run on each one-dimensional plate. For two-dimensional thin-layer chromatography, parallel plates were spotted with mixtures of standards and were developed at the same time as the plates of unknown lipids. In some cases, known and unknown lipids were co-chromatographed. The plates were routinely stained with iodine, ninhydrin, and diphenylamine, or with iodine, ninhydrin, and the molybdate spray, in that order.

The total lipids of *Chloroflexus* were identified by comparison of their R_f values in solvent system (i) with those of known standards run on the same plate and by their behavior on staining the plate successively with iodine, ninhydrin, diphenylamine, and the molybdate spray.

The standard phospholipids used were PE, PC, PS, and phosphatidylinositol (Applied Science Laboratories, Inc.); PG (Supelco., Inc.); and CL (Pierce Chemical Co.).

The MGDG and DGDG of spinach and of *Chlorella vulgaris* were used as glycolipid standards. The total lipids of these tissues were extracted with chloroform-methanol (2:1, vol/vol) at room temperature for several hours. No attempt was made to quantitate the amounts of the cellular lipids.

Fatty acid content of lipids. The fatty acid content of the lipids was determined as described previously (14, 16). The *Chloroflexus* strains were sonicated before saponification in one of the two analyses performed. This did not alter the results obtained. Confirmation of the chain length of the fatty acids was made by hydrogenation of the fatty acid methyl esters and rechromatography, as described previously (14). The position of the double bond in the fatty acids was determined by thin-layer chromatography on silver nitrate-impregnated silica gel thin-layer plates for which development was in toluene at −15 C (9).

Glycolipid analysis. For analysis of the sugars in the glycolipids of the green bacteria, approximately 3

mg of the total lipids of each strain was applied to a thin-layer plate and the lipids were separated by two-dimensional thin-layer chromatography as described above. The lipids were visualized with iodine, and the two glycolipids were eluted from the silica gel with methanol-chloroform (2:1, vol/vol). The solvent was evaporated and the glycolipids were hydrolyzed as described by Constantopoulos and Bloch (8). The resulting sugars were identified by thin-layer chromatography on acetate-impregnated silica gel thin-layer plates using known sugars as references (8).

The glycolipids of *Chloroflexus* were isolated by thin-layer chromatography in solvent system (iii) and further treated as for the glycolipids of the green bacteria. In addition, the sugars were identified by spraying the acetate-impregnated silica gel plates with Glucostat and Galactostat (Worthington Biochemical Corp.). The identification of the sugars was confirmed by thin-layer chromatography on boric acid-impregnated silica gel thin-layer plates (22).

RESULTS

Phospholipids of green bacteria. The phosphate contents of the total lipids of each of the strains of green bacteria are presented in Table 1. Although the purity of the lipid samples was not determined, it is clear that all strains, with the possible exception of strain 2430, contained similar levels of phospholipids.

All seven strains of green bacteria were found to contain significant amounts of phospholipids which exhibited the R_f and staining behavior of CL and PG on silicic acid thin-layer chromatography in four solvent systems. No further analyses of these lipids have yet been performed. No evidence for the ninhydrin-positive lipids PE or PS was found in any of the strains. Unidentified phospholipids were present in all strains. The latter were not characterized definitively (Fig. 1).

Phospholipids of Chloroflexus. Analysis of the lipids in solvent system (i) indicated the presence of PG and PI. The presence of these lipids was not confirmed by other methods. No evidence of PE or PS was found (Fig. 2).

TABLE 1. *Phosphate content of total lipids of green bacteria*

Strain	Phosphate content (μg of phosphate/mg [dry weight] of lipid)
1930	43.8
2430	21.5
2530	31.8
2631	33.6
6030	42.5
6230	43.3
6330	38.9

Glycolipids of green bacteria. All strains were found to contain at least two glycolipids (Fig. 1). These lipids exhibited the same properties as did glycolipids I and II described for *Chloropseudomonas ethylicum* (7). Evidence that these lipids are the same as glycolipid I and glycolipid II was the following. One diphenylamine-positive lipid moved with the R_f of MGDG of spinach and *Chlorella vulgaris* in solvent system (iii) and in the two-dimensional thin-layer chromatography system. The second diphenylamine-positive spot in each strain moved between MGDG and DGDG in both solvent systems. Upon staining with diphenylamine, the spot identified as glycolipid I immediately turned the blue-gray color characteristic of MGDG. The spot identified as glycolipid II first turned brown and then turned blue-gray.

The identities of the glycolipids as glycolipids I and II were confirmed by analysis of the sugars released upon acid hydrolysis of the isolated glycolipids. For each strain, the lipid identified

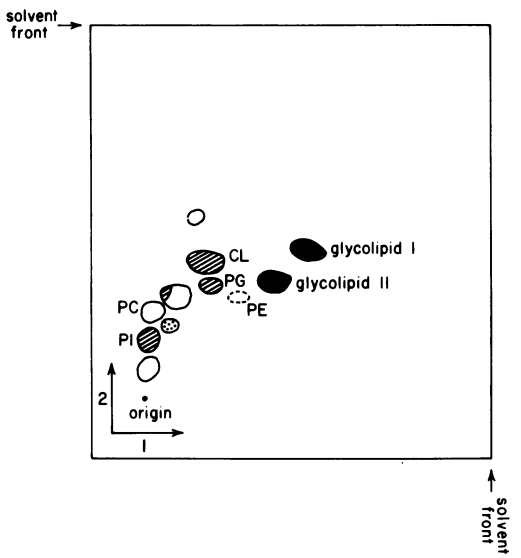

FIG. 1. *Lipids of Chlorobium limicola, strain 6330. The total lipids were separated by two-dimensional thin-layer chromatography in chloroform-methanol-water (65:25:4, vol/vol) (direction 1) and diisobutylketone-acetic acid-water (80:55:15, vol/vol) (direction 2). Symbols: ●, diphenylamine positive; ⊘, molybdate spray positive; ⊛, ninhydrin positive; ○, iodine positive only. Glycolipid I, glycolipid II, CL, and PG were identified as described in the text. The labels PI (phosphatidylinositol) and PC indicate the migration of reference compounds only, although lipid spots were detected at those locations in the unknown sample. ◌ indicates the migration of standard PE. There was no corresponding spot in the unknown sample.*

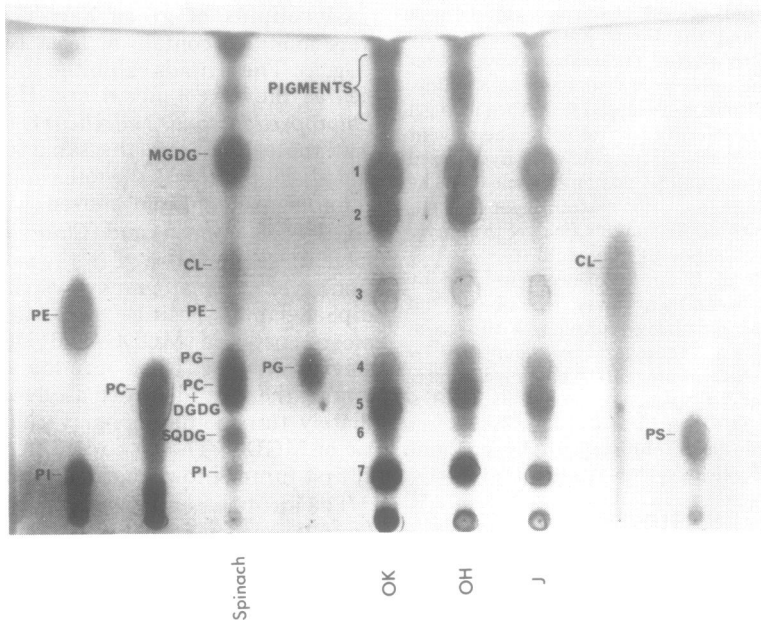

FIG. 2. *Lipids of Chloroflexus aurantiacus. The total lipids of Chloroflexus were separated by thin-layer chromatography in chloroform-methanol-acetic acid-water (85:15:10:3.5, vol/vol) and visualized with iodine. The lipids were tentatively identified as described in the text, (1) MGDG-like; (2) molybdate positive; (3) molybdate and ninhydrin negative; (4) PG; (5) DGDG-like; (6) SQDG?; (7) phosphatidylinositol.*

as glycolipid I was found to yield a single sugar which stained blue-gray with napthoresorcinol as did galactose, and which had the same R_f (0.09) as did galactose. The second glycolipid, identified as glycolipid II, was found to yield one sugar which behaved as galactose (by staining and R_f) and a second sugar which had the same R_f (0.34) and staining behavior with naphthoresorcinol (blue-violet) as did rhamnose.

The presence of SQDG in the lipids of each strain was suggested by the presence of a ninhydrin-, molybdate-, and diphenylamine-negative lipid with the R_f of SQDG in all three one-dimensional thin-layer systems. One or more unknown ninhydrin- and molybdate-negative lipids were also present in each strain (Fig. 1).

Glycolipids of Chloroflexus. All three strains of *Chloroflexus* were found to contain glycolipids which exhibited the same R_f and staining behavior with diphenylamine as did the MGDG and DGDG of spinach. The presence of SQDG in each strain was suggested by the presence of a ninhydrin-, molybdate-, and diphenylamine-negative lipid with the R_f of SQDG (Fig. 2).

Hydrolysis of the two glycolipids of each strain of *Chloroflexus* revealed that both glycolipids contained galactose. Galactose was identified on the basis of R_f in two solvent systems and staining with napthoresorcinol and Galactostat. In addition, both glycolipid fractions yielded a sugar with the R_f of mannose which is Galactostat positive and Glucostat negative. This could be a galactose derivative. A small amount of a Glucostat-positive sugar with the R_f and staining properties of glucose was also detected in the diglycolipid fraction (Fig. 3).

Fatty acids of green bacteria. The fatty acid composition of the extractable lipids of six of the strains of green bacteria is presented in Table 2. All strains contained large amounts of myristic acid, palmitic acid, and hexadecenoic acid.

Thin-layer chromatography of the fatty acid methyl esters of strain 6330 on silver nitrate-impregnated silicic acid thin-layer plates showed that the unsaturated fatty acids of this strain migrated with oleic acid rather than with *cis*-vaccenic acid. This suggests that the double bond of 16:1 is between carbons 9 and 10.

The fatty acid identified as 17cy co-chromatographs with 9,10-methylene hexadecenoic acid from *Escherichia coli* on 10% diethyleneglycol succinate gas chromatography columns,

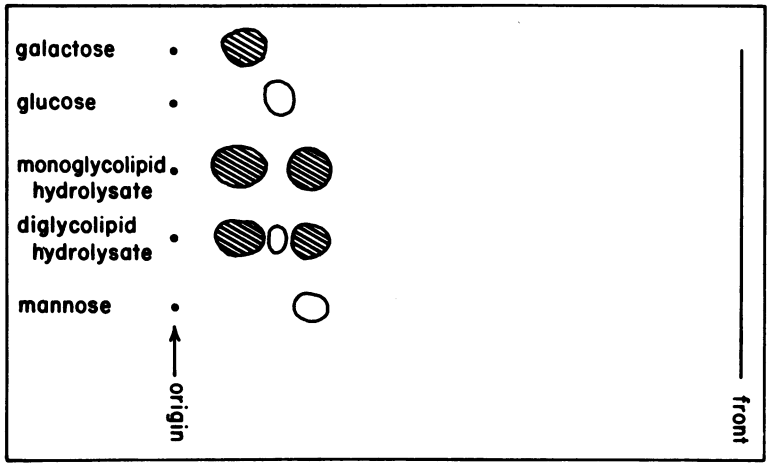

FIG. 3. *Sugars of the glycolipids of Chloroflexus aurantiacus. The hydrolysates of the glycolipids of strain OH-70-fl were analyzed by thin-layer chromatography on acetate-impregnated silica gel. Symbols:* ●, *Galactostat-positive spots;* O, *Glucostat-positive spots.*

TABLE 2. *Fatty acids of green bacteria*

Strain	Per cent of total fatty acids						
	14:0	16:0	18:0	14:1	16:1	17cy	18:1
1930	12	23	1	tr[a]	52	3	2
2430	16	15	tr	1	64	1	1
2530	14	21	tr	1	47	11	tr
2631	10	29	tr	tr	51	2	2
6230	21	10	tr	2	43	21	1
6330	13	17	tr	2	57	3	—

[a] tr, Trace (less than 1%).

is resistant to mild catalytic hydrogenation, and is sensitive to bromination (5). This analysis was made for strains 2530 and 6230 only, since they are the only strains found to contain significant amounts of this acid.

Fatty acids of Chloroflexus aurantiacus. The fatty acid composition of the three strains of *Chloroflexus* is presented in Table 3.

Separation of the total fatty acids of each strain on $AgNO_3$-impregnated plates revealed that fatty acids with double bonds at positions (7, 8), (9, 10), and (11, 12) were present (Fig. 4). Argentation chromatography of 16:1, 17:1, 18:1, 19:1, and 20:1 collected from the gas chromatograph showed the fatty acid isomers which were present in each strain (Table 4). Each fatty acid isomer separated by argentation chromatography was rechromatographed on 10% diethyleneglycol succinate to check its purity. It was then hydrogenated in order to confirm the chain length.

DISCUSSION

The phosphate contents of the total lipids of the green bacteria reported here are all within the same range as the values reported by Cruden and Stanier (10) for strain 6230. The presence of PG and CL in the green bacteria is a property shared with other photosynthetic bacteria. Likewise, the blue-green algae also contain PG. However, the absence of PE distinguishes the lipids of the green bacteria and of *Chloroflexus* from those of the other photosynthetic bacteria, whereas it is a property shared with the blue-green algae (14, 15, 30).

TABLE 3. *Fatty acids of Chloroflexus*

Strain	Per cent of total fatty acids													
	14:0	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	18:2	19:0	19:1	20:0	20:1
OH	tr[a]	tr	tr	12	4	3	3	14	52	2	1	3	1	4
OK	tr	tr	tr	8	3	5	7	10	46	3	2	8	1	5
J	tr	tr	tr	17	2	3	1	27	34	3	1	2	1	5

[a] tr, Trace (less than 1%).

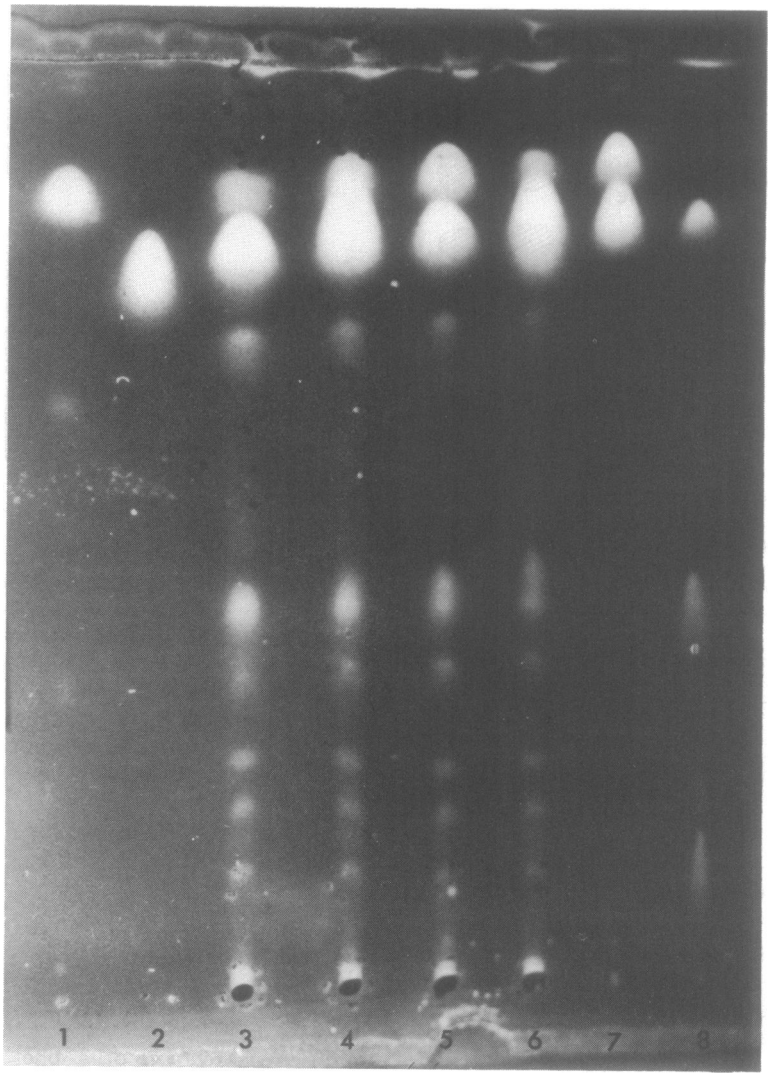

FIG. 4. *Fatty acids of Chloroflexus aurantiacus strain OK-70-fl. The total fatty acid methyl esters were separated by thin-layer chromatography on silver nitrate-impregnated plates. (1) Cis-vaccenic acid; (2) palmitoleic acid; (3) fatty acids of strain OK-70-fl alone; (4) 3 + oleic acids; (5) 3 + cis-vaccenic acid; (6) 3 + palmitoleic acid; (7) palmitoleic acid + cis-vaccenic acid; (8) oleic acid, linoleic acid, α-linolenic acid. Visualization 2, 7-dichlorofluorescein, photographed under ultraviolet light.*

The occurrence of PE in the bacteria has been discussed by Goldfine (11), who points out that PE is nearly a universal component of the lipids of the bacteria, although the distribution of lipids derived from PE, including PC, is less widespread. Our findings suggest that the green bacteria and *Chloroflexus* are taxonomically or evolutionarily more closely related to each other and to the oxygen-evolving blue-green algae than to other bacteria.

The properties of glycolipids obtained from all seven type strains of the green bacteria are the same as those of *Chlorobium thiosulfatophilum*, strains 6130 and 6230, and *Chlorobium limicola*, strain 1230, previously described (10). These glycolipids were originally characterized by Constantopoulos and Bloch (8) after their isolation from *Chloropseudomonas ethylicum*. However, that "strain" has subsequently been shown to be a two-membered culture of *C. limicola* and another nonphotosynthetic bacterium (12). Thus, the source of the glycolipids

TABLE 4. *Position of double bonds in fatty acids of Chloroflexus*

Fatty acid	Double bond position		
	7,8[a]	9,10	11,12
16:1	+[b]	+	−
17:1	−	+	−
18:1	±	+	±
19:1	−	+	+
20:1	−	±	+

[a] This double bond position is inferred.
[b] +, Present; −, absent.

was uncertain in the analyses by Constantopoulos and Bloch.

The occurrence of the galactose-containing glycolipid I in the green bacteria distinguishes this group from all the other photosynthetic bacteria so far examined, and indeed from most other gram-negative bacteria (11). Only in the sulfur purple bacterium *Chromatium* strain D has a monoglycolipid (glucosyldiglyceride) been found in significant quantities. Moreover, the presence of a lipid with similar properties in *Chloroflexus* supports the above conclusion that the green bacteria and *Chloroflexus* are taxonomically rather close to each other and to the blue-green algae.

In extending these observations, although the structure of the glycolipids of *Chloroflexus* has not been examined, the presence of a galactose-containing glycolipid with the chromatographic properties of DGDG as found in the blue-green algae and in photosynthetic eukaryotic cells would suggest that *Chloroflexus* is more closely related to these higher forms than are any of the other photosynthetic bacteria so far described. These conclusions, which are based entirely on the complex lipid patterns of the organisms in question, are borne out by comparison of other properties of the groups (Pierson and Castenholz, submitted for publication).

The cellular localization of the various glycolipids described for photosynthetic microorganisms is known. Glycolipid I, which may be MGDG, is localized in the chlorobium vesicles, whereas glycolipid II is in the cell membrane of green bacteria (10). MGDG and DGDG are known to be localized in the photosynthetic lamellae of a blue-green alga (*Anabaena variabilis*) and in the chloroplasts of eukaryotic photosynthetic organisms (18). The occurrence of other sugars in the glycolipids of a eukaryotic photosynthetic microorganism has been reported, although their cellular localization is unknown (C. N. Kenyon, Ph.D. thesis, Harvard University, Cambridge, Mass., 1967). It would be interesting to know the cellular localization of the galactose-containing glycolipid found in *Chloroflexus* which exhibits a polarity similar to that of DGDG. It would also be of interest to know the structures of the glycolipids of *Chloroflexus*. Are they indeed MGDG and DGDG, perhaps with one galactose occurring as a derivative?

The function of the galactolipids in the photosynthetic organelles is not known. What is known is that the monogalactolipid and chlorophyll contents of the cell are related, suggesting that this lipid has an important structural role in the process of photosynthesis (3). It is striking that this lipid occurs in only those photosynthetic bacteria in which photosynthesis takes place in chlorobium vesicles. Finally, the green bacteria, *Chloroflexus*, the blue-green algae, and eukaryotic photosynthetic organisms are all characterized by chlorophylls with ring structures on the same oxidation level (7; Pierson and Castenholz, submitted for publication).

Examination of the fatty acids of the green bacteria and *Chloroflexus* revealed that both groups contain only the saturated and monounsaturated fatty acids characteristic of the bacteria in general and of some strains of blue-green algae (11, 14, 15). The trace of 18:2 found in *Chloroflexus* was probably derived from the yeast extract in the medium used for growth, since saponification and extraction of yeast extract yielded 18:2.

Any bacterium capable of anaerobic growth which always contains unsaturated fatty acids would be expected to utilize the anaerobic pathway for synthesis of unsaturated fatty acids. The presence of several different isomers of each unsaturated fatty acid as found in *Chloroflexus* is indicative of this pathway.

The presence of 17cy in the green bacteria is rather unusual since these fatty acids are not often found in gram-negative bacteria not belonging to the order *Eubacteriales* (11). Another unusual feature of the fatty acids of *Chloroflexus* is the presence of saturated and monounsaturated odd-chain-length fatty acids. These fatty acids did not co-chromatograph with any of the known common branched-chain fatty acids.

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